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SYMPOSIUM

Heme oxygenase 1 (HMOX1) gene expression in hemodialysed uremic patients+

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ABSTRACT The oxidative stress and the released heme molecule of degrading red blood cells during hemodialysis induce the expression of heme oxygenase 1 gene (HMOX1). The goal of our study was to measure the expression of HMOX1 during dialysis and compare it with the common oxidative markers. We established a cRT-PCR (competitive reverse transcriptase PCR) method to measure the mRNA levels. The mRNA levels were calculated to the white blood cell count respectively for each sample. We measured a 1-5-fold increase in the HMOX1 gene expression after the dialysis session, which dropped back to the original value 48 hours after the dialysis. The base level of HMOX1 mRNA of hemodialysed patients before dialysis was in the range of normal controls. The changes in mRNA levels showed significant correlation with the plasma hemoglobin ($r=0.72$, $p<0.001$) and plasma bilirubin ($r=0.71$, $p<0.002$) changes. We found no correlation between the changes of mRNA levels and other biochemical markers (GSH, GSSG). Our results suggest that the induced HMOX1 expression during hemodialysis is primarily due to the undergoing hemolysis. **Acta Biol Szeged 47(1-4):147-151 (2003)**

KEY WORDS

oxidative stress
hemodialysis
heme oxygenase 1
gene expression
cRT-PCR

Oxidative stress is known to play an important role in the etiology of numerous human conditions, including atherosclerosis, cerebral ischemia, and several neurodegenerative, neuromuscular disorders and renal injury. Cellular antioxidants appear to be crucial for the reduction of ox-idative stress and the prevention of associated pathology. Of the known enzymatic antioxidant systems, perhaps the best characterized are superoxide dismutases, catalases, and glutathione peroxidase, which directly metabolize free radical precursors (Coyle and Puttfracken 1993). A group of stress proteins including heme oxygenase 1 are up regulated during oxidative stress and ostensibly act to maintain the structural and functional integrity of damaged proteins, and thus plays cytoprotective role. Heme oxygenases catalyze the rate-limiting step in heme degradation, resulting in the formation of iron, carbon monoxide, and biliverdin, which is subsequently converted to bilirubin by biliverdin reductase.

Heme is a tetrapyrole with a redox active iron center, and functions as a co-factor for various proteins such as hemoglobin, myoglobin, cytochromes, catalases (Balla et al. 1993; Muller-Eberhard and Fraig 1993). In this role heme is essential for many biological process including oxygen transport and energy production. Tissue damage or cell injury can destabilize heme-proteins and result in "free" hem, which in turn can damage cellular components and disrupt cellular function (Balla et al. 1991a, 1991b; Nath et al. 1995, 1998). Heme itself promotes to the oxidative degradation of DNA (Aft and Mueler 1983), proteins (Aft and Mueler 1984) and amplifies the hydrogen peroxide mediated endothelial cell dysfunction (Balla et al. 1993). The elimination of excess "free" heme is done by the heme oxygenases (HOs). At present three functional isoforms of HO are known (HO-1, HO-2 and HO-3). HO-2 and HO-3 are constitutional form while HO-1 is inducible by heme (Alam et al. 2002), and various other physiological and non-physiological stimuli such as hemin (Shibahara et al. 1978), inflammatory cytokines (Rizzardini et al. 1993; Kutty et al. 1994), UV-irradiation, heavy metals and arsenite (Keyse and Tyrrel 1989). The HO-1 obviously plays a cytoprotective role in oxidative stress (Stocker 1990) and heme mediated injury (Nath et al. 1995, 2000, 2001), which is also confirmed by the knockout animal model (Poss and Tonegawa 1997). Furthermore HO-1 has been implicated in many clinically relevant disease states including atherosclerosis, transplanted graft rejection (Avihingsanon et al. 2002), acute renal injury (Schaaf et al. 2002; Yoneya et al. 2002), as well as others.

The hemodialysed uremic patients suffer from a periodic oxidative stress (three times a week), which occurs due to the cell shearing and hemolysis during HD. The considerable amount of hem damages the vascular endothelium and contributes to atherosclerosis of these patients. This raises the question how the heme oxygenase reacts to this elevated stress condition. The HO-1 gene expression had not been

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studied yet in uremic patients; therefore we wanted to investigate the changes in HO-1 activity simultaneously with the usual parameters of oxidative stress, and the correlation of the two systems during HD. It is known that HO-1 is expressed in monocytes, leucocytes and macrophages (Shibahara et al. 1978) therefore we used blood samples for the non-invasive monitoring of metabolites and HO-1 levels. Since HO-1 induction is primarily regulated at the mRNA level of HMOX1 gene transcription it was feasible to develop a method for measuring the mRNA levels.

Materials and Methods

Seventeen patients with end stage renal disease (ESRD) on regular hemodialysis (HD; 9 male and 8 female aged 14,9±3,1) were studied. The distribution of the original nephrological diagnosis in the ESRD group were as follows chronic pyelonephritis with reflux nephropathy 9, interstial nephritis 2, membranoproliferate glomerulonephritis 3, focal segmental glomerulosclerosis 2, rapidly progressive glomerulonephritis 1. All patients had been on antihypertensive therapy with a combination of angiotensin converting enzyme inhibitor and calcium channel blocker because of hypertension. The patients had been on 4-h bicarbonate HD (3 times a week) for 1-5 years. HD was performed with hemophan single-use dialyser. The blood flow rate was 200 ml/min and the dialysate flow rate was 500 ml/min. During HD heparin was used as an anticoagulant, with initial dose of 500 IU, followed by continuous infusion rate of 1000 IU/h. The venous blood samples were collected before, immediately after HD and 48 hours following HD. For mRNA separation 1ml native blood were collected and stabilized in RNA/DNA stabilization Reagent for Blood/Bone Marrow (Roche Diagnostics GmbH, Germany) for biochemical assays 2-2 ml of EDTA and heparinised blood were collected.

Plasma hemoglobin, bilirubin and blood carboxyhemoglobin, methemoglobin levels

For the assay of plasma hemoglobin and bilirubin contents, heparinised plasma samples were diluted 1:40 (v/v) with 5 mmol L^{-1} PBS, pH 7.4, and measured spectrophotometrically according to the method of Winterbourn (1979). Blood carboxyhemoglobin concentration was also measured spectrophotometrically (OSM 3 Hemoximeter, Radiometer, Coppenhagen).

Reduced and oxidized glutathione

Highly sensitive and specific determinations of total and oxidized glutathione concentrations were made by combining previously accepted standard methods (Németh and Boda 1994).

For the assay of total glutathione concentration, 25 µl of whole blood anticoagulated with EDTA was immediately hemolyzed in 2.5 ml cold (4°C) 0.01 M potassium phosphate buffer containing 5 mM EDTA, pH 7.5, and stored at –70°C until further use. For analysis, 25 µl of hemolyzate was added to the standard glutathione assay mixture (final volume 1 ml) in the order indicated: DTNB (0.6 µmol), glutathione reductase (10 µg) and NADPH (0.2 µmol). The combined action of DTNB and NADPH in the presence of glutathione reductase results in a reaction cycle, the rate of which depends on the total concentration of GSH and GSSG recorded spectrophotometrically at 412 nm during the first 6 min. For the determination of GSSG blood was hemolyzed in the phosphate buffer described above previously supplemented with 0.02 mM N-ethylmaleimide (NEM). The reaction of NEM with GSH results in the formation of a stable complex, preventing GSH from participating in the enzymatic assay and from being oxidized to GSSG. As NEM is an inhibitor of glutathione reductase, it was necessary to separate it by gel filtration with Sephadex G-10 before further analysis. The concentrations of the thiols were expressed with reference to hemoglobin (hgb) determined by the cyanmethemoglobin method.

RNA extraction and cRT-PCR experiments

mRNA was extracted from 1ml venous blood using mRNA Isolation Kit for Blood/Bone Marrow (Roche Diagnostics GmbH, Mannheim, Germany). To avoid loss of mRNA due

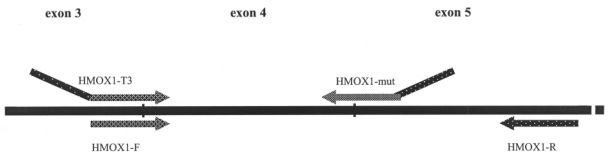

Figure 1. Part of HMOX1 CDS from exon 3 to 5, HMOX1-T3 and HMOX1-mut were used to create a PCR product from which we transcribed our control RNA, which sequence only differs in a 20bp deletion from wild type HMOX1 mRNA. The mRNA and control RNA was reverse transcribed and consecutively amplified by the same primers HMOX1-F and HMOX1-R. Primers were designed to span exon boundaries to avoid binding at genomic DNA level.

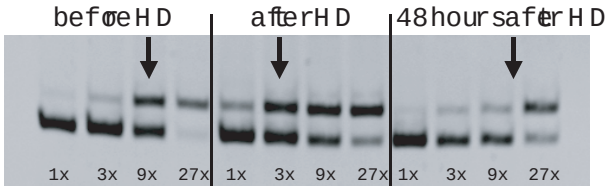

Figure 2. HMOX1 mRNA expressions of a patient with oxidative hemolysis; before, after and 48 hours after HD. The 1, 3, 9, 27x dilutions of control RNA was reverse transcribed with equal amounts of mRNA and amplified by the same PCR primers. The arrows show the concentrations where mRNA concentration is equal to the control RNA concentration.

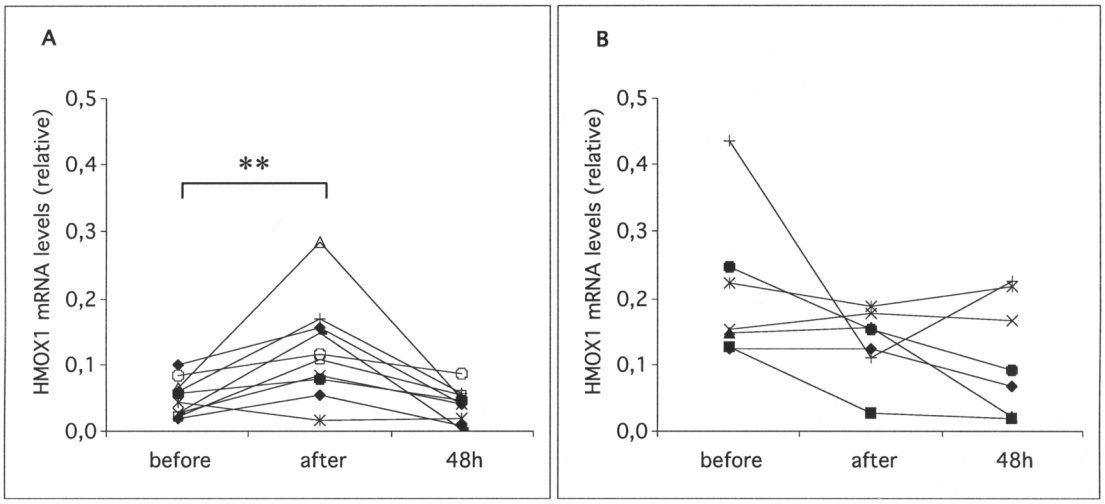

Figure 3. HMOX1 mRNA expression A) in patients with low HMOX1 levels B) in patients with elevated HMOX1 expression.

to limited glass surface or ligands we scaled the separation as we were separating 1.5ml blood samples. Competitive RT-PCR was used to identify the expression of heme oxygenase 1 gene (HMOX1). The competitor RNA was created by in vitro mutagenesis and transcription with T3 RNA polymerase (Fermentas AB, Vilnius, Lithuania) according to Waha et al. (1998). The primers to generate the competitor were as follows: HMOX1-T3: 5' AAT TAA CCC TCA CTA AAG GGA GAC GTT TCT GCT CAA CAT CCA GCT C 3' and HMOX1-mut: 5' CCT GGG AGC GGG TGT TGA GTG GGG GGC AGA ATC TTG CAC TTT G 3'. First strand cDNA was generated using RevertAid™ First Strand cDNA Synthesis Kit (Fermentas AB, Vilnius, Lithuania) using specific primer of HMOX1-R. The specific oligonucleotide primers used for the PCR reaction were designed to span exon boundaries thus binding only at the cDNA level (Fig. 1). The PCR amplification were carried out by the following program: initial denaturation at 94ºC for 5 min followed by 25 cycles of denaturation at 94ºC for 20 sec, annealing at 61ºC for 30 sec and extension at 72°C for 20 sec followed by a final extension at 72°C for 10 min. The primers were as follows: HMOX1-F: 5' CGT TTC TGC TCA ACA TCC AGC TC 3' and HMOX1-R: 5' CCT GGG AGC GGG TGT TGA GTG G 3'. The amplified cDNAs were examined on 6% polyacrylamide gel and stained with ethidium bromide. The target heme oxygenase 1 band was estimated by the ratio to the competitor by densitometry (AlphaImager™, AlphaEase 5.5). The HMOX1 mRNA concentrations were expressed with reference to white blood cell count; since we hadn't calculated the copy number of our control RNA they represent relative values (we used the same dilutions of control RNA). Since control RNA and mRNA was handled in the same tubes together their relative ratio remained the same throughout the whole process.

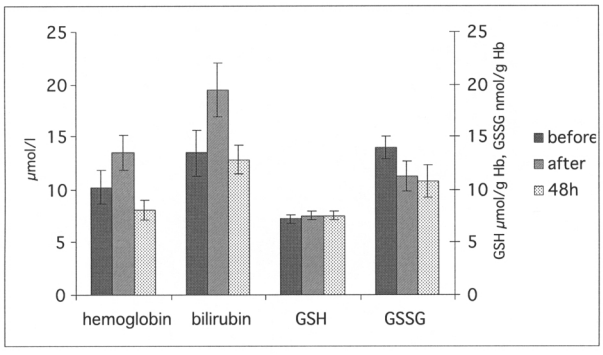

Figure 4. Changes in metabolite levels in the patients (n=10) with low HMOX1 mRNA levels before HD.

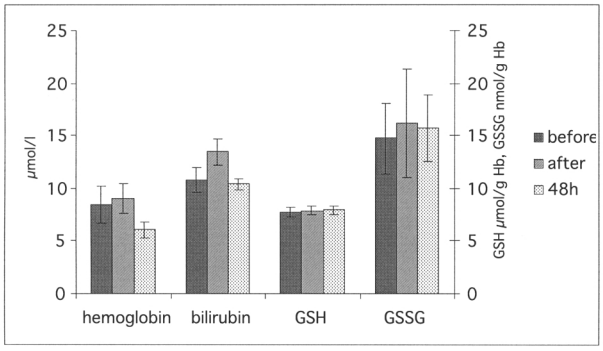

Figure 5. Changes in metabolite levels in the patients (n=7) with elevated HMOX1 mRNA levels before HD.

Statistical analysis

Clinical data on the patients are reported as means ± SD (x ± SD), whereas results of biochemical analyses are shown in figures as means ± standard errors (x ± SEM). Statistical analyses were performed with the Tukey test, Student's *t*-test, and correlation analysis. The significance level for all tests was taken as p=0.05.

Results

We established a cRT-PCR method for the measurement of HMOX1 mRNA levels from blood (Fig. 2). We measured the plasma hemoglobin, plasma bilirubin, carboxyhemoglobin, methemoglobin, GSH and GSSG levels from venous blood samples.

We divided our patients into two groups; in the first group of patients (n=10) the HMOX1 mRNA levels were low before HD. In this group we measured a 1-5-fold increase (p=0.0014) in the HMOX1 gene expression after HD, which dropped back to the original value 48 hours after HD (Fig. 3A). The plasma hemoglobin and bilirubin levels increased after HD and their value dropped back 48 hours after HD (Fig. 4). In the second group there was already a higher level of HMOX1 mRNA expression, in these patients the HMOX1 was not induced further after HD (Fig. 3B). In this group of patients the plasma bilirubin levels increased after HD, and the plasma hemoglobin levels remained the same after HD but both of them decreased 48 hours later (Fig. 5). In the two groups there were no significant changes in the GSH and GSSG levels (Fig. 4,5).

The mRNA levels varied greatly between individuals therefore we calculated the quotient of after HD/before HD of the measured metabolite and HMOX1 mRNA levels, which gives the effect of HD on these parameters.

We found significant correlation between the changes of HMOX1 mRNA levels and plasma hemoglobin (r=0.72, p<0.001) levels, plasma bilirubin (r=0.71, p<0.002) levels compared before and after HD (Table 1, Fig. 6). We found no significant correlation between the changes of HMOX1 mRNA levels and changes in the measured oxidative stress markers (Table 1).

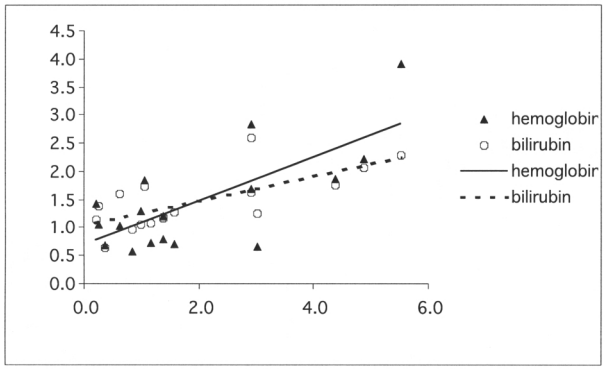

Figure 6. Correlation of the changes in HMOX1 mRNA levels with the changes in hemoglobin, bilirubin levels in all patients (n=17) before and after HD.

Discussion

The average duration of a session for a chronic uremic patient is 4 hours. This time is enough to induce HO-1. Since HO-1 is induced at the mRNA levels the earliest response to the HO-1 induction can be monitored by the measurement of mRNA levels. For the quantitative mRNA analysis we had chosen a cRT-PCR method. This PCR based technique permits the precise analysis from small amount of samples (Kozbor et al. 1993; Waha et al. 1998). The analysis of HMOX1 mRNA from blood samples is noninvasive and easily conducted at the hemodialysed patients. This also gives the advantage that the target tissue (blood) is the same for the measurement of mRNA and other parameters.

In the patients who had already elevated HMOX1 expression before HD the HO-1 catabolyzed the "free" hem and was not further induced. In the patients who had low mRNA levels before HD the HMOX1 was induced by the undergoing hemolysis. The great differences between the base HMOX1 mRNA levels in the patients can be explained by the different redox or inflammatory states (Nath et al. 2001).

Due to the shearing of cells and hemolysis during HD the plasma hemoglobin levels increased, which according to our data and the literature (Nath et al. 2000, 2001; Alam et al. 2002) induced HO-1. In those patients where no marked hemolysis occurred there was no significant HO-1 induction. We did not find any significant changes in the GSH and GSSG levels due to the undergoing HD that confirms our previous data (Túri et al. 1991) and suggest that the glutathione system is not elevated in these conditions.

The effect of HD could be best monitored by the measured parameters after HD and before HD.

The significant correlation between the changes of hemoglobin (inductor) and bilirubin (sequential product) levels with HMOX1 mRNA levels before and after HD shows that hemoglobin induced HO-1, and the induced HO-1 catabolyzed the hem-molecules.

Since we found no correlation between HMOX1 gene

Table 1. Correlation between the changes of biochemical parameters and HMOX1 mRNA levels (r).

biochemical markers	HMOX1 mRNA before HD/after HD
Plasma hemoglobin	0.72; p<0.001
Plasma bilirubin	0.71; p<0.002
GSH	-0.314
GSSG	-0.254
GSH/GSSG	-0.177

expression and the common oxidative markers (GSH, GSSG) our results suggest that the induced HMOX1 expression during hemodialysis is primarily due to the occurring hemolysis.

By our study it is clear that this group of patients has an inducible HMOX1 gene that responds to the stimuli of its substrate and is functionally intact (the induced HMOX1 expression leads to elevated bilirubin level). As we have no hemodialysed control people we don't know whether the induction due to hemolysis in our patients is comparable to normal people. Therefore further studies are needed to investigate a promoter polymorphism – which directly alters the inducibility of HMOX1 gene (Yamada et al. 2001)– on a control population and hemodialysed patients to see whether the HO-1 plays a role in the pathomechanism of vascular damage in uremic patients.

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